Proteomic analyses of the acute tissue response for explant rabbit corneas and engineered corneal tissue models following in vitro exposure to 1540 nm laser light

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ABSTRACT

Two-dimensional electrophoresis and histomorphometry were used to determine if equivalent protein changes occurred within native rabbit corneas and engineered corneal tissue models following in vitro exposure to single pulse, 1540 nm laser light operating at a pulse width of 0.8 milliseconds. Frozen sections of exposed tissues were processed to detect laser-induced protein changes. Isoelectric points, molecular weights and relative densities were used to characterize corneal proteins of interest that were then identified using MALDI-MS peptide fragment analysis. Increasing radiant exposures of corneal tissues were associated with progressively more severe necrosis of the epithelium and stroma in both the native and engineered tissues.

Keywords: Rabbits, cornea, laser, infrared laser, histomorphometry, proteomics

1. INTRODUCTION

Infrared lasers have wavelengths where water is generally considered the principal chromophore. Most of the energy from an infrared laser light exposure to the eye is deposited in the cornea because the cornea is approximately 80% water. In a transparent tissue, such as the cornea, minimal scattering occurs and the absorption factor is principally associated with light attenuation (1, 2). If sufficient energy is present, tissue damage occurs initiating the process of corneal wound healing. Proteins play an important role in corneal tissue dynamics as either indicators or mediators of laser-tissue interactions. Previous studies by our research group demonstrated that damage to rabbit corneal tissue produced by a single pulse exposure of 1540 nm infrared laser light was associated with an increased activity of matrix metalloproteinases in remodeling the extracellular stromal matrix (3). The present study extends these observations through the development of engineered tissue models and novel proteomic analysis methods.

2. MATERIALS AND METHODS

Experimental Animals

All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, University of Illinois and were conducted in strict accordance with the "Guiding Principles for the Care and Use of Research Animals." Rabbit eyeballs were obtained from animals undergoing terminal procedures not related to this study and without clinical manifestations of ocular disease. Immediately after euthanasia of the animal and enucleation of the eye, each eyeball was rinsed in 0.1 M phosphate buffered saline and transferred to F12/DMEM media (MediaTech) supplemented with 10% NuSerum (Collaborative Biomedical), 2 mM L-glutamine, 500 IU/ml penicillin, 500 μg/ml streptomycin and 1.25 μg/ml amphotericin. Enucleated eyeballs were maintained in F12/DMEM media at 4°C until used for laser exposure within 24 hours of enucleation.

Engineered Rabbit Corneal Tissue

Explant rabbit eyeballs served as the source of native corneal tissue used to isolate corneal epithelial cells and stromal keratocytes. Cultures of rabbit corneal epithelial cells and keratocytes were expanded in culture and served as the seed cultures for the engineered tissues. Corneal tissues were produced in two steps. First, a liquid collagen/keratocyte seed culture suspension was added to a Transwell (Costar) polycarbonate tissue culture insert contained within a 12 well tissue culture plate. The polycarbonate membrane of the insert served as a platform for the gelatinization of the stromal collagen and the growth of stromal keratocytes. The collagen/keratocyte suspension forms a gel during incubation

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(37°C, 5% CO_2) and the tissues grown in culture for 3-5 days. Second, a seed culture suspension of corneal epithelial cells was plated upon the collagen/ keratocyte gel and grown in culture for an additional 7-14 days. The tissue culture fluid level was slowly lowered over the incubation period until an epithelial cell-air interface was established. Under optimum culture conditions, the epithelial layer will stratify into basal, wing and superficial cells.

Laser Exposures

All laser exposures were single pulse, 1540 nm laser light set at 0.8 millisecond pulse widths with a spot size of 0.0008 cm^2. The laser had an Erbium:Glass rod and was manufactured by Laser Sight Technologies (Winter Park, FL). The pulse widths were measured using a Germanium detector (Thor Labs PDA 255) connected to a TDS 644B-digitizing oscilloscope (Tektronix). Energy measurements were made with a EPM 2000 detector (Molectron) connected to a J-25 energy probe (Molectron). Energy densities ranged from 40-100 J/cm^2 at $1/e^2$.

Histomorphometry

After overnight incubation (37°C in 5% CO2 and 95% humidity), the corneas were frozen in OCT embedding medium (Tissue-Tek) and frozen sections were taken using a motorized cryomicrotome (Bright Instruments). All tissue sections for histomorphometric analysis of laser effects were taken through the middle of the lesion. Images of histologic sections were obtained using a Leitz Orthoplan microscope equipped with a SpotRT digital camera (Diagnostic Instruments). Alterations in the epithelial parameters (e.g., area of hyaline coagulative change vs. area of granular coagulative change) and stromal parameters (e.g., area of coagulative necrosis, number and distribution of keratocytes) were evaluated.

Proteomic Analysis

Tissue isoelectric focusing (TIF) was conducted as reported by Eurell and Meachum (4). Frozen sections of corneal tissues between twenty-five and one hundred and fifty microns were obtained and placed on a pH 3-10 gradient polyacrylamide gel (CleanGel, Amersham Biosciences) containing appropriate ampholytes and 8M urea. The first dimension electrophoresis was performed at a constant temperature of 15°C using an isothermal controlled electrophoresis chamber (Model FB1001, Fisher) and a crossover regulated power supply (Model EC650, EC, Inc.). These first dimension gels were cut into strips and placed on top of 4-20% gradient SDS-PAGE mini-gels (8cm x 8cm) (Model 260, Hoefer Sci). The second dimension electrophoresis was conducted at a constant current of 40 milliamps per gel using a crossover regulated power supply (Model EC650, EC, Inc.). After the 2D-PAGE electrophoresis, gels were stained using a mass spectroscopy compatible silver stain kit (SilverQuest, Invitrogen). Digital images of silver-stained gels were captured using a digital gel documentation system (EDAS, Kodak) and stored for calibration against commercial isoelectric point and molecular weight standards (Invitrogen). Individual protein spots were excised from gels following the 2D-PAGE method listed above and processed by a sequential exchange of buffers followed by overnight incubation with sequencing grade trypsin (Sigma), extraction and desalting of the tryptic peptides (ZipTip, Millipore). Identification of individual protein "spots" in the two-dimensional gel was conducted using Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry.

3. RESULTS AND DISCUSSION

Quantitative alterations in the epithelial parameters (e.g., area of hyaline coagulative change vs. area of granular coagulative change) and stromal parameters (e.g., area of coagulative change) were directly related to the laser power applied to the tissue. Markedly similar cellular responses were seen between the native and engineered corneal tissues. Using histomorphometric criteria, the engineered tissues developed in this study provided an appropriate model for acute tissue damage following laser exposure.

Conventional proteomic methods involve tissue homogenization, electrophoretic protein separation and mass spectrometry. Although these techniques provide extensive data on the total protein composition of a tissue, they give no information on protein distribution in the different cellular compartments of a heterogeneous tissue such as the cornea. We have developed a novel proteomic method using histologic sections as the tissue source in conjunction with tissue isoelectric focusing (TIF). TIF is a direct recovery procedure and does not require homogenization or extraction of the specimen. The major advantage of this approach is that protein profile evaluation can be made with reference to the corresponding structural detail in a tissue section. TIF methods are particularly useful in laser-tissue interaction studies because the laser causes a small, discrete lesion that is surrounded by normal tissue. We believe that the homogenization

and extraction steps in standard proteomic methods would have difficulty separating the protein "signal" from the background "noise." With the TIF procedure, we can section through the lesion with micron precision and explore the tissue response in far-lesion, near-lesion and central-lesion tissue zones.

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